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It Depends on How You Read It: Alternative Translations of a Single RNA Message

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X-ray Determination of isomeric forms of 2S-4,4,4-Trifluorovaline

We have determined the single crystal X-ray structures of the HCl salt forms of 2S-4,4,4-trifluorovaline to confirm our stereochemical assignments. The optical rotations for 2S,3S- and 2S,3R-Tfv are +7.2 (c 0.75, 1 N HCl) and +12.8 (c 0.5, 1 N HCl) respectively. The crystallographic data for each structure have been deposited to the Cambridge Crystallographic Data Centre (CCDC) with registration numbers 229994 and 229993. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

Synthetase Cloning, Expression and Purification

The cloning of *E. coli* isoleucyl-tRNA synthetase (IleRS) was described in reference 1; the resulting plasmid is designated as pQE-ileS. A similar cloning scheme was used to clone *E. coli* valyl-tRNA synthetase (ValRS). *E. coli* genomic DNA was prepared by using the DNEasy kit from Qiagen (Chatsworth, CA). The ValRS gene was amplified from genomic DNA by PCR using the following primers: 5'-GCT CAA CCT GAA TAC GGA GCT CTG GAA AAA TGG-3' (valSSac1fw); 5'-GGG AGT TAT GCC TTC TTG GTA CCA TTT TCT GTA AGA G-3' (valSKpn1bw). *Pfx* polymerase was used for PCR to ensure high fidelity amplification. The resulting 2900 base pair DNA fragment was ligated into the expression plasmid pQE31 through the *Sac*I and *Kpn*I sites to yield pQE-valS. The integrity of the cloned gene was confirmed by DNA sequencing. The cloned synthetase has the N-terminal leader sequence MRGSHHHHHHTDPHASSGK.

pQE-ileS and pQE-valS were individually transformed into the XL-1 blue strain of *E. coli* (Stratagene) to afford the expression strains XL[pQE-ileS] and XL[pQE-valS] respectively. Synthetase expressions were conducted in LB media and induced at OD_{600}=0.6 with 1 mM IPTG. After three hours, the cells were harvested and lysed by sonication. The enzymes were purified using Ni-NTA agarose resin under native conditions according to the manufacturer's protocol. Residual imidazole was removed on an ion-exchange column and proteins were eluted into Tris buffer (50 mM Tris-HCl,

pH=7.4, 1 mM DTT)/50% wt glycerol. Aliquots were stored at –80 °C. The concentrations of the purified proteins were measured by absorbance at 280 nm under denaturing conditions.

Amino Acid Activation Assays

IleRS and ValRS activities were evaluated by the amino acid-dependent ATP-PP_i exchange reaction as described previously.[2,3] The assay measures the formation of $^{[32]}$P-labeled ATP by the enzyme-catalyzed exchange of $^{[32]}$P-pyrophosphate (PP_i). In a 200 μL reaction buffer (50 mM HEPES (pH 7.6), 20 mM $MgCl_2$, 1 mM DTT, 2 mM ATP and 2 mM [^{32}P]-PP_i (0.5 TBq/mol, in $NaPP_i$ form)), enzyme was added to a concentration of 100 nM and amino acid to a concentration of 3 μM to 10 mM (dependent on the rate of activation of the amino acid). At successive time point, aliquots (20 μL) were quenched into 500 μL quench solution (200 mM PP_i, 7% w/v $HClO_4$ and 3% w/v activated charcoal). The charcoal was washed twice with 10 mM PP_i and 0.5% $HClO_4$, and the absorbed ATP was counted via liquid scintillation counting. Kinetic parameters (k_{cat} and K_m) were determined by non-linear curve fitting of the data to a Michaelis-Menten model. The results reported in Table 1 are averages from duplicate experiments.

Plasmid Construction for *in vivo* Assays of Codon Assignment

The IleRS and ValRS genes along with their endogenous promoters were amplified directly from *E. coli* genomic DNA. The primers used for IleRS were: 5'-GCC GGT ATT CGC CAG CTA GCT GGA AGT GCA TTT G-3' (ileSNhe1fw); 5'-GAG GAT CAG GTA TTT GCT AGC CCA GAT CGA TAA TCA G-3' (ileSNhe1bw). The primers used for ValRS were: 5'-GTC TGC GAA CAA GCT AGC AGA TTT TGC CAC-3' (valSNhe1fw); 5'-CCA GAT AAA GGC TTG CTA GCC AGT ATT TCA CGG G-3' (valSNhe1bw). The PCR conditions were 50 ng/100 μL of template DNA, 300 ng/100 μL of each primer, 95 °C denaturing temperature (0.5 min), 55 °C annealing temperature (1 min), 68 °C extension temperature (3 min). The resulting DNA fragments were digested by *Nhe*I and ligated into pQE15 (Qiagen) through the *Nhe*I site (pQE15 encodes murine dihydrofolate reductase (mDHFR) under control of a bacteriophage T5 promoter). The orientation of the inserts was checked by restriction enzyme digestion; plasmids containing the inserted genes with the same coding direction as the ORFs of mDHFR were selected. This orientation ensures that the transcription terminator sequence in the vector will be placed immediately downstream of the IleRS or ValRS gene. The resulting vectors were denoted pQE15-ileS and pQE15-valS respectively. The isoleucine and valine double auxotrophic strain AIV-IQ[1] was individually transformed with pQE15-ileS and pQE15-valS to yield the expression strains AIV-IQ[pQE15-ileS] and AIV-IQ[pQE15-valS] respectively.

***in vivo* Translational Assays of Codon Assignment**

M9 medium supplemented with glucose (0.2 wt%), $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM), thiamine (5 mg/L), 20 natural amino acids and antibiotics (ampicillin and chloramphenicol) was inoculated individually with overnight cultures of expression strains AIV-IQ[pQE15-ileS] and AIV-IQ[pQE15-valS]. When OD_{600} reached 0.8-1.0, the cultures were sedimented by centrifugation at 4 ^{o}C and the cell pellets were washed twice with 0.9% NaCl solution (sterilized). The cells were then resuspended in M9 minimal medium as described above, but without one natural amino acid (**1** or **2**) and supplemented with **3** or **4** at a concentration of 250 mg/L. After 10 min incubation, IPTG was added to a concentration of 1 mM to induce protein expression. Cells were harvested by centrifugation after 4 hours and lysed in buffer A (8 M urea, pH=8, 100 mM NaH_2PO_4, and 10 mM Tris). After a freeze/thaw cycle, the samples were subjected to SDS-PAGE analysis to evaluate protein expression. Samples of the test protein mDHFR were purified by nickel-affinity chromatography on Ni-NTA spin columns according to the manufacturer's instructions (Qiagen).

Amino Acid Identification at Valine and Isoleucine Codon Sites

Amino acid analysis, MALDI-MS and LC-MS/MS analysis of tryptic peptides were employed to identify the amino acids at the codon sites of interest. For amino acid analysis, the purified protein samples in 8 M urea were subjected to buffer exchange against water by ultrafiltration (Millipore, MWCO=5000) and submitted to the Molecular Structure Facility at the University of California at Davis for analysis. Quantitation was carried out based on standard chromatograms of the HCl (6 N) hydrolysis products.

For MALDI-MS analysis, 10 μL of protein sample in elution buffer (8 M urea, pH=8, 100 mM NaH_2PO_4, 10 mM Tris-HCl) was added to 90 μL of NH_4OAc (50 mM) solution. Modified trypsin (2 μL, Promega, 0.2 mg/mL) was added and the mixture was digested overnight. Trifluoroacetic acid (TFA) was used to quench the reaction (pH<4.0) and the sample was subjected to chromatography on a $ZipTip_{C18}$ column (Millipore). The peptides were eluted into 3 μL 50% CH_3CN, of which 1 μL was added into 10 μL matrix solution (α-cyano-β-hydroxycinnamic acid, 10 mg/mL in 50% CH_3CN). The samples were analyzed on an Applied Biosystems Voyager DE Pro instrument.

LC-MS/MS analysis of tryptic peptides was carried out on a Finnigan LCQ ion trap mass spectrometer with HPLC pump and ESI probe. The purified tryptic peptide solution (3 μL) (eluted from a $ZipTip_{C18}$ column as above) was diluted with 25 μL of distilled water and injected into the HPLC. Peptides were separated on a C18 HPLC column and eluted at a flow rate of 30 μL/min using a linear gradient of 0-60% of solvent B (CH_3CN) in solvent A (H_2O with 0.1% TFA) over 45 min. The column eluent was directed to the electrospray source and tandem mass sequencing was carried out by fragmentation of the precursor ion with m/z corresponding to the desired tryptic fragments.

References

(1) Wang, P.; Tang, Y.; Tirrell, D. A. *J. Am. Chem. Soc.* **2003**, 125, 6900-6907.

(2) Hendrickson, T. L.; Nomanbhoy, T. K.; Schimmel, P. *Biochemistry* **2000**, *39*, 8180-8186.

(3) Tang, Y.; Tirrell, D. A. *Biochemistry* **2002**, *41*, 10635-10645.

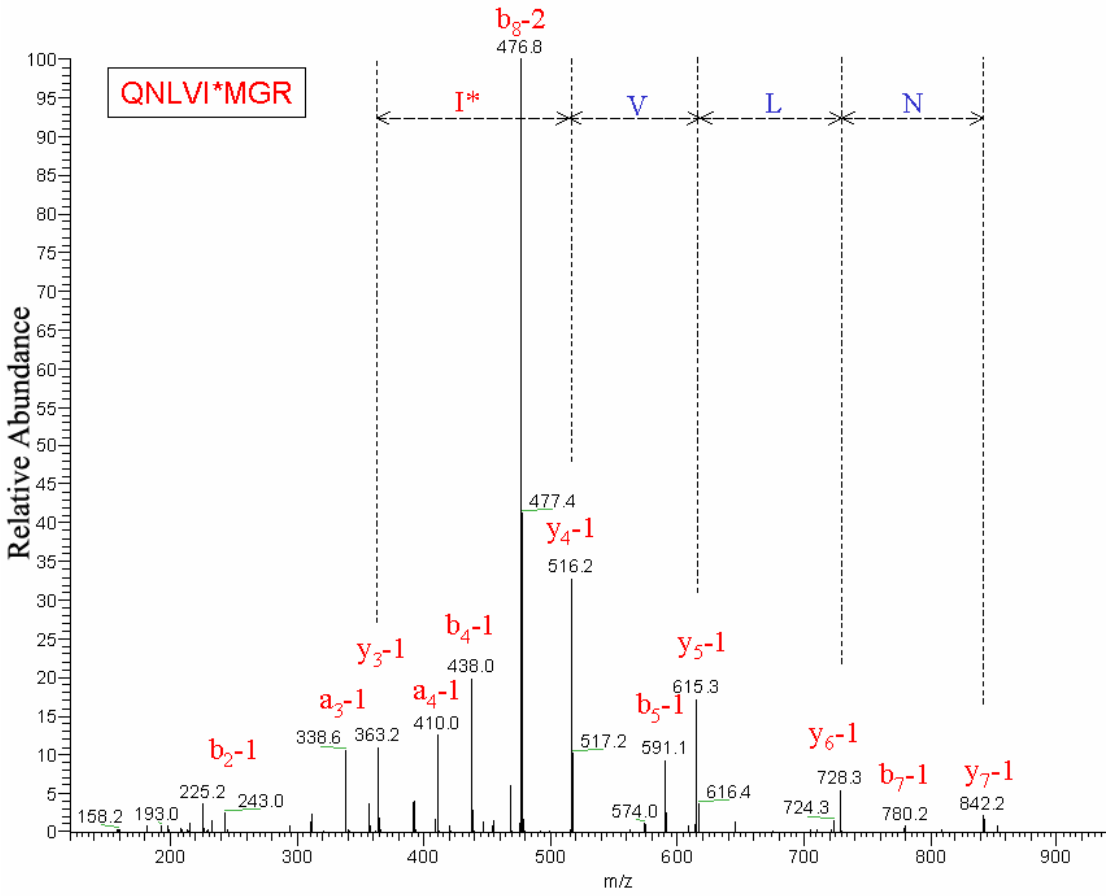

Figure S-1:

Tandem mass spectrum of the peptide (QNLVI*MGR) derived from mDHFR expressed in media supplemented with **4** (250 mg/L). mDHFR was expressed in an isoleucine and valine double auxotrophic strain (AIV) transformed with pQE-ileS. The partial sequence NLVI* can be assigned from the annotated y ion series; I* is unambiguously assigned as **4**.

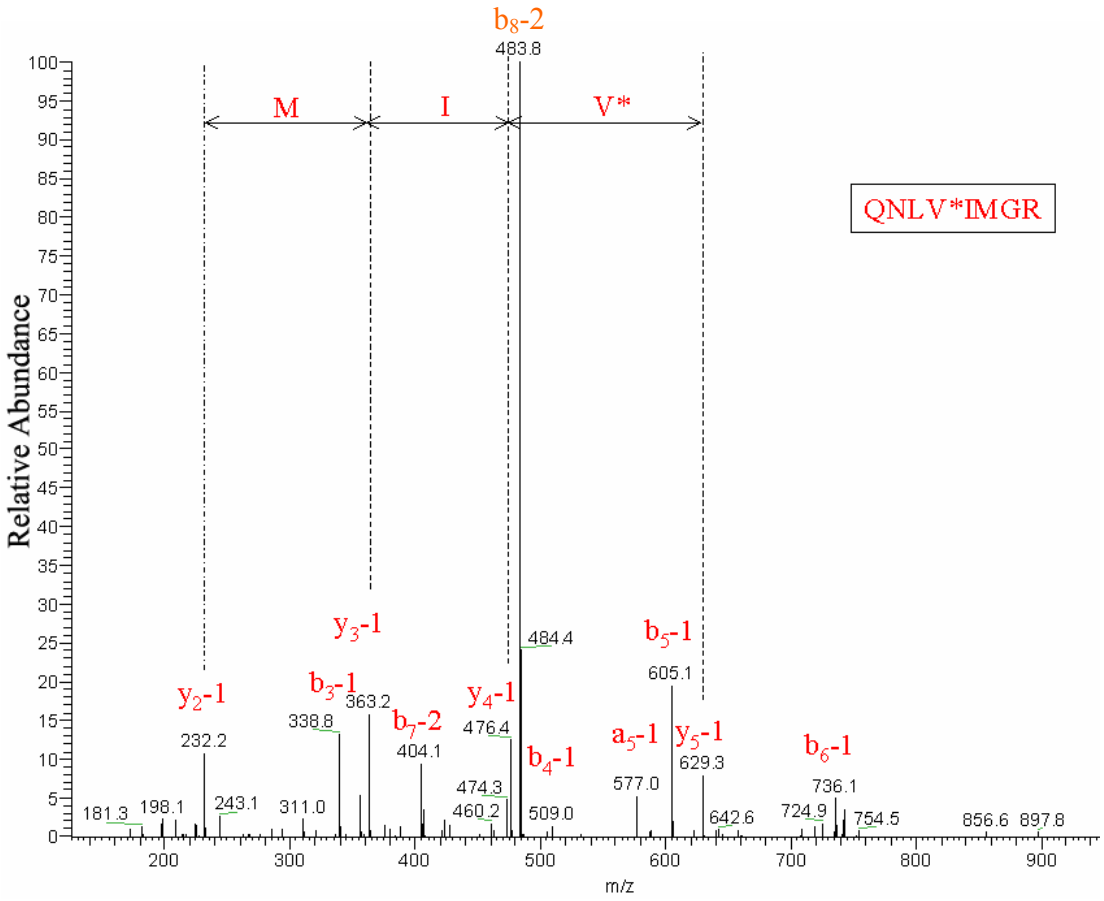

Figure S-2:

Tandem mass spectrum of the peptide (QNLV*IMGR) derived from mDHFR expressed in media supplemented with **4** (250 mg/L). mDHFR was expressed in an isoleucine and valine double auxotrophic strain (AIV) transformed with pQE-valS. The partial sequence V*IM can be assigned from the annotated y ion series; V* is unambiguously assigned as **4**.

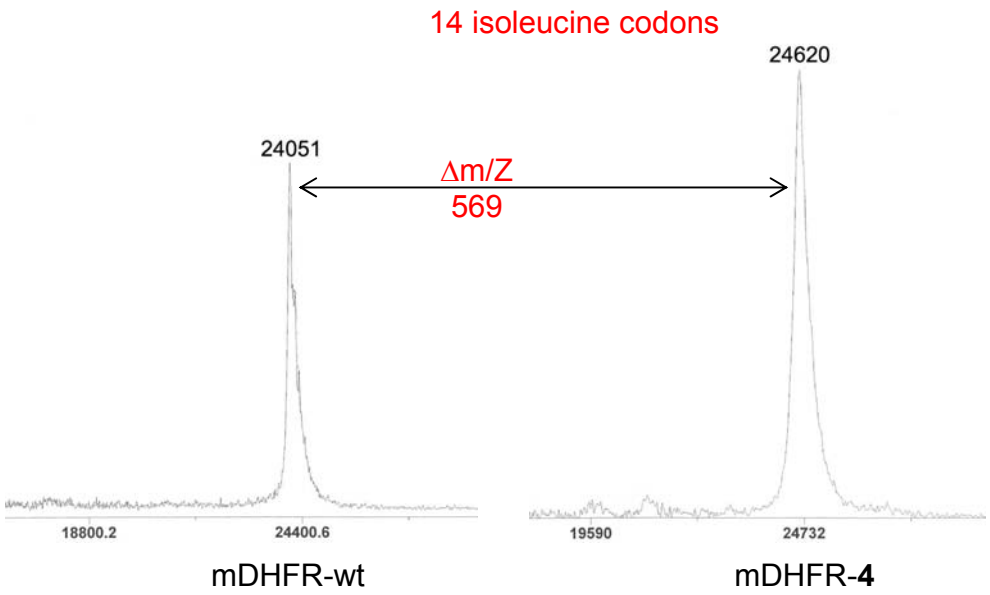

Figure S-3:

MALDI spectra of mDHFR expressed in media supplemented with **4** (250 mg/L). mDHFR was expressed in an isoleucine and valine double auxotrophic strain (AIV) transformed with pQE-ileS. mDHFR-wt (wild-type protein) served as a control.

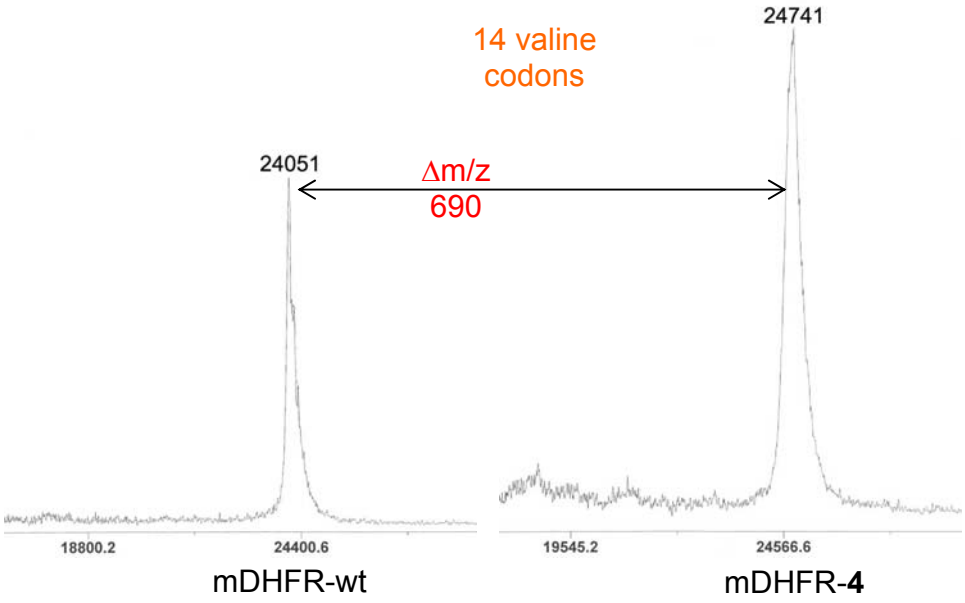

Figure S-4:

MALDI spectra of mDHFR-**4** expressed in media supplemented with **4** (250 mg/L). mDHFR was expressed in an isoleucine and valine double auxotrophic strain (AIV) transformed with pQE-valS. mDHFR-wt (wild-type protein) served as a control.